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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 9/00, 47/32, 47/38		A1	(11) International Publication Number: WO 94/20070
			(43) International Publication Date: 15 September 1994 (15.09.94)
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(22) International Filing Date: 11 March 1994 (11.03.94)			
(30) Priority Data:		(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KG, KP, KR, KZ, LK, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
08/029,668 11 March 1993 (11.03.93) US			
08/119,578 13 September 1993 (13.09.93) US			
(60) Parent Applications or Grants			
(63) Related by Continuation			
US 08/119,578 (CIP)			
Filed on 11 March 1993 (11.03.93)			
US 08/029,668 (CIP)			
Filed on 13 September 1993 (13.09.93)			
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(54) Title: POLYMERIC MUCOADHESIVES IN THE DELIVERY OF IMMUNOGENS AT MUCOSAL SURFACES			
(57) Abstract			
A pharmaceutical composition for inducing an immune response against an infectious agent in an animal comprises an antigen against the infectious agent and a mucoadhesive, and optionally an adjuvant, to boost the immune response to the antigen in the animal. Preferably, the antigen is derived from an influenza virus. The pharmaceutical composition is useful in a method of immunizing an animal by oral administration of the composition to the animal.			

DESCRIPTIONPOLYMERIC MUCOADHESIVES IN THE
DELIVERY OF IMMUNOGENS AT MUCOSAL SURFACESCROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application Serial No. 08/119,578, filed September 13, 1993 (Attorney docket No. 05060-0003-01000), which is a continuation-in-part of application Serial No. 08/029,668, filed March 11, 1993, (Attorney Docket No. 05060-0003-00000), now abandoned. The entire disclosure of each of these applications is relied upon and incorporated by reference herein.

BACKGROUND OF THE INVENTION

Oral administration of immunogens is cost effective, safe and encourages compliance. It is now well established that stimulation of immunocompetent tissues and cells of the small intestine results in stimulation of protective immune responses at other mucosal surfaces through the common mucosal immune system. In some cases, such stimulation also results in serum responses. However, the use of oral vaccination is currently limited in medical practice to a small number of vaccines.

Typically, oral immunization requires significantly higher dosages of immunogen for the elicitation of a protective immune response, and some vaccines fail to elicit a response when delivered orally. In addition, attempts to increase the safety of the vaccines by attenuation and the use of synthetic and subunit preparations usually result in antigen preparations which are less efficacious than the whole intact antigen.

Various adjuvants, including muramyl dipeptide and fluoride, have been used in conjunction with experimental

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oral vaccines in attempts to increase the immune response by stimulating the ability of the immune cells to respond. An alternative approach to adjuvants is to "package" the antigens in a particulate form and thus to present the antigen in a form that is readily taken up by the cells of the immune system. For example, antigens incorporated into, or attached to, polymeric microparticles, nanoparticles, or liposomes are frequently more immunogenic than soluble antigens. These particulate antigens may be more effective because they become trapped in the mucus or because they are selectively scavenged by the M cells of the gut mucosa.

The immune response after oral immunization can also be increased by using immunogens that selectively bind to epithelial cells (e.g., the hemagglutinin of influenza, *Vibria cholerae*) or by coupling immunogens to compounds that selectively bind to these cells (e.g., the adhesins of enteric bacteria such as *Shigella* or *Pseudomonas*; toxoids such as cholera or pertussis toxoid; or pollen grains).

Nevertheless, there still exists a need in the art for methods and compositions for enhancing immune responses to immunogens administered at mucosal surfaces.

SUMMARY OF THE INVENTION

The object of the present invention is to enhance immune responses to immunogens administered at mucosal surfaces. In one embodiment of this invention the immune response after immunization by oral and other mucosal routes is induced or increased by associating the immunogen with polymeric mucoadhesives. The theoretical and practical value of mucoadhesives and other bioadhesives in drug delivery (particularly by transdermal and buccal devices) is well known in the art, but the use of an immunogen with a mucoadhesive to enhance an immune response was heretofore unknown.

In another embodiment of this invention the immune response after immunization by oral and other mucosal routes is induced or increased by including an adjuvant with the

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immunogen and polymeric mucoadhesive. The effectiveness of adjuvants in oral delivery of an immunogen in combination with a mucoadhesive was previously unknown.

DETAILED DESCRIPTION

It is known in the pharmaceutical arts that subcutaneous injection of immunogen into a host can produce an acceptable immune response. In addition, mucosal administration and particularly oral administration of immunogen can produce immune response in some cases, although often suboptimal ones. The immunizing composition and immunization method of the invention make it possible to enhance the immune responses in a host when the immunogen is administered at mucosal surfaces of the host. Using this mode of administration this invention has made it possible in some cases to achieve a systemic immune response more nearly comparable in magnitude and duration to the response that is obtained when the same antigen is administered to the host by subcutaneous injection, while achieving a stronger mucosal immune response than by either subcutaneous injection or by mucosal administration of antigen alone.

This invention can be carried out with immunization compositions containing any of the conventional immunogens known for human or veterinary use to stimulate immune response in a host. As used herein, the term "immunogen" means a substance that when introduced into the body stimulates humoral, mucosal, or cell-mediated immunity. The terms "immunogen" and "antigen" are used interchangeably herein.

As used herein, the term "immune response" means the development of specifically altered reactivity to foreign antigens following exposure to an immunogen, as indicated by an increase in mucosal or serum antibodies against the immunogen, or by a measure of cell-mediated immunity such as cytokine activity or proliferation.

The immune response produced by this invention is conveniently determined by measuring change in mucosal

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immunity, such as a change in salivary IgA measured by ELISA, or by determining change in systemic immunity, such as a change in antibody levels by ELISA, or by determining change in cellular immunity, such as by measuring T-cell proliferative responses. In the case of immunization against influenza virus, measurement of serum hemagglutinin inhibition titer (HI) is also useful. In the case of influenza, this invention makes it possible to obtain an immune response in mice that is about equal in magnitude to the immune response obtained in about the same period of time after subcutaneous injection of the same materials in equivalent amounts when these analytical techniques are employed.

The classes of immunogens that can be used to stimulate the common mucosal immune system after oral or other mucosal route of immunization according to this invention include without limitation: 1) any infectious disease antigens that are capable of inducing an immune response when administered to mucosal surfaces, including particularly the natural mucosal immunogens (for example, influenza virus and antigens of enteric bacteria such as *Escherichia coli*, *Vibrio cholerae* and *Helicobacter*), which may bind to the mucosal epithelium through specific interactions between such antigens and ligands and receptors on the mucosal membrane; 2) live attenuated enteric pathogens (for example, polio virus or rotavirus); 3) synthetic particulate antigens (for example, antigen-containing microspheres and nanospheres); 4) allergens which bind to the mucosae (for example, ragweed pollen); and 5) autoantigens and tissue antigens (for example, myelin basic protein, CD4, melanoma specific protein).

The immunogen preparation, which may be the entire immunogen, a modified immunogen, a synthetic immunogen or subunits of the above, can be derived from a virus (for example, influenza, HIV, rotavirus or hepatitis), a bacteria (for example, *Shigella*, *Bordetella pertussis* or *Chlamydia*), a

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eukaryotic parasitic organism (for example, Plasmodium, the causative organism of malaria), a toxin (for example, cholera toxin or endotoxin), an allergen (for example, ragweed pollen) or a tissue marker (for example, melanoma, CD4, or myelin basic protein).

In a preferred embodiment of this invention, the immunogen is derived from a virus. This invention is applicable to a wide variety of viruses against which a vaccine is desired. The invention is especially well suited for use against the common respiratory or enteric viruses. For illustrative purposes, the influenza virus is described here. However, the invention is not intended to be limited to the influenza virus, nor to the strain of influenza virus used in the embodiments described herein. Other viruses against which the invention can be employed include, for example, parainfluenza virus, respiratory syncytial virus, rhinovirus, corona virus, and adenovirus.

In the case of influenza virus, the choice of virus from which a vaccine is derived will depend in part on the strain or subtype of influenza against which protection is desired. In order to obtain a vaccine useful against a particular strain of influenza, it is preferable to use as the starting material of the vaccine a strain of influenza that possesses at least one antigenic determinant in common with the strain against which the vaccine is to be utilized.

It will be understood that this invention can be employed with any animal influenza virus type and subtype, as well as different strains of a given subtype. The influenza virus will typically be one that infects animals, such as chickens or ducks, and pigs. The invention is particularly suitable for use in primates, including humans. Thus, for example, the invention can be carried out with human influenza subtypes HON1, H3N8, H2N2 (pandemic Asian virus), H3N2 (pandemic Hong Kong virus), H1N1 (pandemic Russian virus), or other viral subtypes caused by antigenic shift, as well as subtypes resulting from antigenic drift. An example

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of a strain of a given subtype used in this invention is influenza virus A/Udorn/307/72 (H3N2), which was a gift from Dr. B. R. Murphy (National Institutes of Health, Bethesda, MD). This subtype is a human influenza virus strain that also infects squirrel monkeys and mice. The A/Udorn/307/72 subtype is well suited for use in experiments in animals, such as mouse infection models.

In the present invention, mucoadhesives are employed with the immunogen to increase the efficacy of delivery of the immunogen to the mucosal immune system. Various mucoadhesives are currently used in the pharmaceutical industry for drug delivery, as film formers and as viscosity increasing agents, and in the food industry, and are generally considered to be safe by the Food and Drug Administration. Examples include: sodium carboxymethyl cellulose, carbopol, polycarbophil, sodium alginate, and hydroxypropyl methyl cellulose.

The inventors believe that for oral immunization the interaction of a mucoadhesive with the mucin layer allows the mucoadhesive and any associated immunogen to bind to the mucin layer that is associated with all gut mucosae. This association may increase the efficacy of the interaction of the associated compound with the mucosal membrane by: (1) removing the compound from the central lumen of the gut to the relatively immobile mucus layer associated with the mucosal epithelium, thus increasing the time span in which the compound has the potential of interacting with the immune cells of the mucosal membrane of the intestine and its immunocompetent cells; (2) increasing the effective concentration of the compound by separating it from the large volume of fluid and matter in the lumen into the relatively thin layer of mucin adjacent to the mucosal membrane; and (3) protecting the immunogen from denaturation by recovery into this mucin layer, where the molecules are protected from the pH changes and catabolic enzymes in the gut by the diffusion barrier of the polymeric mucin. The interaction of the

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immunogens with the mucoadhesive polymers may protect the immunogen from the effects of phase changes and the detergent action of bile salts. Finally, the hydrophilic properties of the mucoadhesives result in the dehydrated mucoadhesive removing water from its immediate environment. This dehydration of the mucosal epithelial layer may result in increased bioavailability of the immunogen. In addition, association of the immunogen with the polymer may stabilize the immunogen. While these factors may explain the effectiveness of mucoadhesives, the inventors do not wish to be restricted by the accuracy of these explanations.

The known mucoadhesives are polymers which bind to the polymeric, negatively charged mucin through one or several mechanisms, including hydrophobic interactions, van der Waal forces, interaction of charged groups, polymer admixing and linear chain association, binding of specific residues and interaction of receptors and ligands. All known mucoadhesives are polymers, and it is taken for granted in the literature, but a monomeric mucoadhesive might be possible.

Mucoadhesives are generally not in themselves immunogenic and many are safe for human use. They are currently used as mucomimetic agents, as surgical glues, as wound healing adjuncts, in antidiarrheal and anti-constipation agents, and for the delivery of drugs. Mucoadhesives have been successfully applied to transdermal delivery systems for drugs, including peptide drugs, for nasal delivery of drugs (for example, diuretics and insulin) and for controlled and localized delivery of drugs in the buccal cavity (for example, steroids). Although mucoadhesives have been used as depot adjuvants in parenteral immunization and have been studied as potential controlled release systems for the delivery of peptide drugs, they have not been used previously for the mucosal delivery of vaccines.

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Examples of compounds that are considered to act as mucoadhesives include:

- Sodium carboxymethylcellulose
- Poly(acrylic acid)
- Tragacanth
- Poly(methyl vinyl ether co-maleic anhydride)
- Poly(ethylene oxide)
- Methylcellulose
- Sodium alginate
- Hydroxypropylmethyl cellulose
- Karya gum
- Methylethyl cellulose
- Soluble starch
- Gelatin
- Pectin
- Poly(vinyl pyrrolidine)
- Poly(ethylene glycol)
- Poly(vinyl alcohol)
- Poly(hydroxyethylmethacrylate)
- Hydroxypropylcellulose
- Carbopol
- Polycarbophil

These mucoadhesives can be employed in the composition and method of this invention. The preferred mucoadhesives are sodium carboxymethyl cellulose and other cellulosics, polycarbophil, and carbopol. Sodium carboxymethylcellulose is a particularly preferred mucoadhesive. It will be understood that mixtures of these mucoadhesives can also be employed.

The immunization composition of the invention can also include an adjuvant in an amount sufficient to enhance the magnitude or duration of the immune response in the host, or to enhance the qualitative response in the subject, such as by stimulating antibodies of different immunoglobulin classes than those stimulated by the immunogen. The adjuvant should efficiently elicit cell-mediated and humoral immune responses

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to antigens without systemic or localized irritation of the host system. Preferably, the adjuvant has low pyrogenicity. Well known adjuvant formulations for human or veterinary applications can be employed. Such adjuvants can be based on emulsions, with or without mycobacteria, or adjuvants based on adsorption of antigens to aluminum salts, especially aluminum hydroxide or aluminum phosphate. Among these adjuvants are oil adjuvants based on mineral, animal and vegetable oils. Oil based adjuvants are useful for increasing humoral responses of farm animals to the vaccine antigens, and certain oil-based adjuvants have been tested for human use. Typical adjuvants are Freund's complete adjuvant and Freund's incomplete adjuvant.

Suitable adjuvants that have been developed more recently, include liposomes, immune-stimulating complexes (ISCOMs), and squalene or squalene emulsions. Surface active agents having adjuvant activity can also be employed. These include saponin-like Quil A molecules in ISCOMs and Pluronic® block copolymers that are used to make stable squalene emulsions. Saponins are surface-active agents widely distributed in plants.

Analogues of muramyl dipeptide (MDP) or muramyl tripeptide (MTP), such as threonine analog of MDP and lipopolysaccharide (LPS) having adjuvant activity and reduced side effects, are also suitable for use as adjuvants. LPS has been found to produce good effects in mice. Synthetic analogs of MDP and the monophosphoryl derivative of lipid A are also known for their adjuvant activity and reduced pyrogenicity. Another suitable peptide is the synthetic muramyl peptide MTP-PE (N-acetyl-muramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-[1,2-dipalmitoyl-sn-glycero-3-(hydroxyphosphoryloxy)] ethylamide). A particularly suitable formulation is Syntex Adjuvant Formulation-1 or SAF-1, which combines the threonyl analog of MDP in a vehicle comprised of Pluronic L-121 triblock polymer with squalene and a small proportion of Tween 80® as an emulsifying detergent. The preferred adjuvants for use in

humans are MDP and its analogs, with or without squalene, saponins, and the monophosphoryl derivative of lipid A.

Other compounds have been found to have adjuvant properties when given orally together with antigen. These adjuvants include ox bile, polycations, such as DEAE-4 dextran and polyarnithine, sodium dodecylbenzene sulphate, lipid-conjugated materials, streptomycin, vitamin A, and other agents that can influence the structural or functional integrity of the mucosal surface to which they are applied.

When an adjuvant is combined with the immunogen and mucoadhesive in the composition and method of the invention, a further enhancement in immune response is observed. Once again, while not being bound by any theory of operation, the mucoadhesive may facilitate presentation of the immunogen and adjuvant to responding cells to thereby enhance the effects of these substances in a synergistic manner.

Mucosal surfaces to which mucoadhesives may be used to administer antigen include the gastrointestinal mucosae (including stomach, small intestine, large intestine, colon and rectum); oral mucosae (including the buccal and esophageal membranes and tonsil surface); respiratory mucosae (including nasal, pharyngeal, tracheal and bronchial membranes); genital (including vaginal, cervical and urethral membranes); and ocular membranes. Preferred routes of administering the composition of the invention to a host are oral, nasal, rectal, and swab on the tonsil. Oral administration is the particularly preferred mode of administration because of its simplicity and because it is relatively non-invasive. It will be understood that any of the modes of administration can be combined. For example, the initial dosage can be given orally and a booster dosage given nasally, or vice versa.

It will be understood that the immunization composition of the invention can also be employed in a vaccine. The vaccine can be a therapeutic material containing as the immunogen an antigen derived from one or more pathogenic

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organisms, which on administration to man or animal will stimulate active immunity and protect against or reduce the intensity of infection by these or related organisms. The vaccine can be a live vaccine, an inactivated vaccine, an attenuated vaccine, or a subunit or mixed vaccine.

The composition of the invention can be incorporated into any suitable delivery system. For example, the antigen, mucoadhesive and adjuvant can be combined with a pharmaceutically acceptable liquid vehicle, such as water, buffered saline or edible animal or vegetable oil. The composition can be combined with one or more suitable pharmaceutically acceptable excipients or core materials, such as cellulose, cellulose derivatives, sucrose, gelatin, Starch 1500, NuTab, lactose, malto-dextrin, talc, Cabosil, magnesium stearate, alginate, Actisol, PEG 400, Myvacet, Triacetine, syrup, oil, sorbitol, mannitol, and Plasdone. This list is not intended to be exhaustive or limiting; alternative or additional excipients or core materials can also be used.

It will also be understood that the compositions of the invention can be formulated to include chemical agents that are capable of neutralizing stomach pH. Suitable neutralizing agents include H₂ antagonists, bicarbonate of soda, calcium carbonate, and aluminum hydroxide.

The composition of the invention can be utilized in the form of elixirs, solutions, suspensions, syrups, aerosols, and the like. The composition can also be prepared in dosage units suitable for oral administration, such as particles, granules, beads, tablets, hard gelatin capsules, and soft gelatin capsules.

The immunization composition can be treated to protect the antigen, mucoadhesive, and adjuvant from degradation prior to reaching the target site. In one embodiment, each dosage unit, such as a tablet or capsule, is coated with an enteric coating using conventional methods, including but not limited to pan coating; fluidized bed coating, such as the

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Wurster method or top spraying; spray drying; and an emulsion or microencapsulation method. Optionally, a subcoating can be used, such as hydroxypropyl methyl cellulose, Opadry or Dryclean. An enteric coating is applied to each dosage unit. The enteric coating can be any pharmaceutically acceptable material that protects the antigen and permits release of the antigen in the intestine. The choice and amount of the subcoating or enteric coating is within the ordinary skill in the art, and will depend in part on the dosage form, e.g., tablet or capsule. Examples of suitable enteric coatings are HPMCP 55 (hydroxypropyl methyl cellulose phthalate), CAP (cellulose acetate phthalate), Eudragit, Aquateric, Coateric, Surlease, shellac, and wax.

The immunogen, mucoadhesive, and adjuvant are employed in a combined amount to provide an immune response against an infectious agent. This can be determined by estimating seroconversion, that is, the levels of antibody before and after immunization. If the host has a preexisting antibody titer to the antigen, the success of immunization can be determined by the extent of increase in the level of specific antibody. In cases where there is no correlation between seroconversion and protection, cell-mediated immune response can be monitored.

The amount of antigen, mucoadhesive, and adjuvant per dosage unit will depend on the desired dose and the frequency of administration. In the case of a composition consisting of influenza as the antigen, carboxy methyl cellulose as the mucoadhesive, and muramyl dipeptide as the adjuvant, a single dosage unit can contain

the antigen in an amount of about $10\mu\text{g}$ to about $150\mu\text{g}$ of hemagglutinin (HA), preferably about $5\mu\text{g}$ to about $45\mu\text{g}$ of HA;

the mucoadhesive in an amount of about $10\mu\text{g}$ to about 1g , preferably about 1mg to about 50mg ; and

the adjuvant in an amount of about $1\mu\text{g}$ to about $2\mu\text{g}$, preferably about $10\mu\text{g}$ to about $200\mu\text{g}$.

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The relative proportions by weight of antigen:mucoadhesive: adjuvant in this case is about 5-45: 1000-50000: 0-200. The precise composition will necessarily vary depending on the antigen, adjuvant, and mucoadhesive selected, the species to be immunized, and other factors, and it is within the capacity of one with ordinary skill in the art to search for an optimal formulation. A booster dose can comprise the antigen in an amount sufficient to enhance the initial immune response. It has to be adapted to each protocol depending on the antigen and the host. Multiple doses may be more appropriate for children and for individuals with no known prior exposure. In one embodiment, each dosage unit contains an amount of influenza antigen effective to protect the animal against disease following exposure to the virus.

The dose can be defined as the amount of immunogen necessary to raise an immune response in an individual. For example, the level of homologous neutralizing antibody in the serum is predictive of the susceptibility of an individual to infection by a homologous influenza strain. A serum hemagglutination inhibition titer of about 1:32 or 1:40 or greater is considered to be protective against natural infection by homologous virus. Thus, a preferred dose of the influenza antigen is one that will result in a serum hemagglutination titer of about 1:32 or 1:40 or higher, as determined by standard methods.

The protective effect of the immunogen can also be expressed in terms of the rise in the level of serum hemagglutinin inhibition or mucosal antibody titers that are found post-immunization. In the treatment of influenza, a four-fold increase in the serum HI titer over a period of 7-21 days after immunization is considered to be protective. A four-fold increase in the mucosal antibody titer (e.g., IgA in saliva or nasal wash) over a period of 7-21 days is also considered by some to be protective against influenza. Thus, a preferred dose of the immunization composition according to the invention raises the specific antibody levels in a human

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to these ranges. Immunization dosages can be adjusted until a detectable antibody titer, and preferably a neutralizing antibody titer is obtained.

It will be understood that this invention can be employed with immunogens against any parasitic, bacterial or virus type or subtype, as well as different strains of a given subtype. The parasite, bacterium or virus will typically be one that infects animals, such as dogs, cats, poultry, pigs, horses, and cattle, and especially mammals, such as primates, including humans. The immunogen can be administered before or after the mucoadhesive or adjuvant, but generally the immunogen, mucoadhesive, and adjuvant are simultaneously administered to the subject.

The immunogenic composition of the invention can be prepared by simply mixing the immunogen, mucoadhesive and adjuvant together without covalent bonding or otherwise coupling the ingredients together. The composition thus has the added advantage of ease of preparation.

This invention will now be described in greater detail in the following Examples.

EXAMPLE 1

Vaccine Antigen

The influenza virus A/Udorn/307/72(H3N2), BK6, Egg3, clone 3A (7-25-89) was a gift from Dr. B. R. Murphy, (NIH, Bethesda, MD). This virus was passaged once in embryonated chicken eggs and allantoic fluid stored as stock virus (infection titer 2.53×10^7 plaque forming units (p.f.u.) per 0.2 ml) at -130°C . Ten-day-old embryonated chicken eggs (400-500) were infected with 0.1 ml of stock virus diluted 1:1000 in L-15 medium supplemented with SPG (2.18 M sucrose, 0.038 M KH_2PO_4 , 0.072 M K_2HPO_4 and 0.049 M monosodium glutamate). After incubation for 48 hours at $35 - 36^{\circ}\text{C}$, the allantoic fluid was collected and clarified by centrifugation at $3700g_{av}$ for 20 min. The virus was then collected from the supernatant by centrifugation at $1000,000g_{av}$ for 45 min. The

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pellets were left overnight in 0.5 ml phosphate-buffered saline (PBS) and 0°C, diluted to 6 ml with PBS, vigorously mixed and aggregates removed by centrifugation at $1300g_{av}$ for 15 min. This procedure was repeated three times. After the third centrifugation, the pellet was sonicated and again centrifuged. The pooled supernatants containing the virus suspension were loaded on top of 10-60% continuous sucrose gradients in PBS and centrifuged for 2 hrs. at $1000,000g_{av}$ in a swing-out rotor. The virus bands were collected, diluted 1:1 in PBS and the virus inactivated by incubation with formalin (1:4000 v/v) for 72 hrs. at 37°C. The material was dialyzed overnight at 4°C against PBS, pelleted as above, resuspended in PBS or water to 5 mg protein ml^{-1} and stored at -80°C. Protein was measured by a Coomassie blue binding assay (Pierce, Rockford, IL, USA) for sodium hydroxide-disrupted virus.

Preparation of the Vaccine with Mucoadhesives

Gels were prepared for all the mucoadhesives following simple methods available from the manufacturers. The sodium carboxy methyl cellulose (CMC) used for this experiment was purchased from Aqualon (Wilmington, DE). A CMC gel was prepared by making a 2% solution in H_2O . Carbopol and Polycarbophil acrylic polymers were purchased from B.F. Goodrich (Cleveland, Ohio). Gels were produced from these polymers by preparing a 0.25% suspension of Carbopol in H_2O , and a 0.5% suspension of Polycarbophil in H_2O . The pH of these solutions was raised from approximately 3 to approximately 4 by adding several drops of 1N NaOH. The acrylic polymer suspension becomes a gel when the pH reaches 4. Sodium alginate was purchased from Kelco, a division of Merck & Co., Inc. (San Diego, CA). A gel was produced by preparing a 2% solution in H_2O . Zilactin was purchased from Zila Pharmaceuticals, Inc. (Phoenix, AZ). A 1:10 solution of Zilactin to H_2O was prepared immediately before immunization.

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Influenza vaccine solution (50 μ g of virus protein/10 μ l of phosphate buffered saline) was mixed with the mucoadhesive gels at a 1:50 ratio of vaccine to gel (10 μ l vaccine solution and 490 μ l mucoadhesive per dose). In all cases, mixing was accomplished by simple agitation for approximately 1-2 minutes, until a homogenous solution was achieved by visual inspection, and was done immediately before immunization.

Immunization of Mice

Groups of five BALB/c mice (8 weeks old, female) obtained from Charles River or Jackson Laboratories were used. The vaccine in mucoadhesive (500 μ l) was administered intragastrically with an animal feeding needle. For one control group the influenza vaccine, in 0.1 M NaHCO₃ solution, was also delivered intragastrically with an animal feeding tube.

Systemic immunization of one control group was performed with free antigen (50 μ g/mouse) in a saline solution by subcutaneous route.

Collection of Samples

Blood was collected from the tail veins of mice before and at selected times after immunization. Blood was centrifuged and plasma was collected and frozen. Stimulated saliva was collected with capillary tubes after intraperitoneal injection of mice with carbamyl-choline chloride (1 μ g/mouse). Amounts of 2 μ g each of soybean trypsin inhibitor, phenylmethyl-sulphonyl fluoride, sodium azide and fetal calf serum were added before clarification and storage at -80°C.

ELISA

For determination of antigen-specific antibodies ELISA was performed in 96-well polystyrene microtitre plates (Dynatech, Alexandria, VA, USA) coated with purified A/Udorn

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influenza virus at a concentration of $4 \mu\text{g ml}^{-1}$. Endpoint titres of serum and saliva were determined using horseradish peroxidase-labelled goat IgG against mouse Ig or IgA (Southern Biotechnology Associates, Birmingham, AL, USA) and substrate 2,2'-azino-bis-(3-ethylbenzthiazoline) sulphonic acid (Sigma, St. Louis, MO, USA). The colour developed was measured in a Vmax photometer (Molecular Devices, Palo Alto, CA, USA) at 414 nm.

HI Assays

Haemagglutination inhibition (HI) reaction was performed with mouse sera diluted 1:5 with PBSA and treated for removing non-specific inhibitors (heated by 56°C for 30 min; incubated with 15% acid-treated kaolin for 30 min; and incubated with 10% suspension of chicken red blood cells for 30 min). Twofold dilutions of sera were prepared in 96-well microtitre plates. Viral suspension (8 HA units in an equal volume) was added to each well and incubated at room temperature for 30 min. A 0.5% suspension of chicken erythrocytes was added to each well and incubated at room temperature for 45-60 min. The HI titers were expressed as the reciprocal of the highest dilution that completely inhibited haemagglutination of erythrocytes.

EXAMPLE 2

Vaccine Antigen

The influenza virus A/Udorn/307/72(H3N2), BK6, Egg3, clone 3A (7-25-89) was a gift from Dr. B.R. Murphy, (NIH, Bethesda, MD). This virus was passaged once in embryonated chicken eggs and allantoic fluid stored as a stock virus (infection titer 2.53×10^7 plaque forming units (p.f.u.) per 0.2 ml) at -130°C . Ten-day-old embryonated chicken eggs (400-500) were infected with 0.1 ml of stock virus diluted 1:1000 in L-15 medium supplemented with SPG (2.18 M sucrose, 0.038 M KH_2PO_4 , 0.072 M K_2HPO_4 and 0.049 M monosodium

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glutamate). After incubation for 48 hours at 35-36°C, the allantoic fluid was collected and clarified by centrifugation at 3700g_{av} for 20 minutes. The pellets were left overnight in 0.5 ml phosphate buffered-saline (PBS) and 0°C, diluted to 6 ml with PBS, vigorously mixed and aggregates removed by centrifugation at 1300g_{av} for 15 minutes. This procedure was repeated three times. After the third centrifugation, the pellet was sonicated and again centrifuged. The pooled supernatants containing the virus suspension were loaded on top of 10-60% continuous sucrose gradients in PBS and centrifuged for 2 hours at 100,000g_{av} in a swing-out rotor. The virus bands were collected, diluted 1:1 in PBS and the virus inactivated by incubation with formalin (1:4000 v/v) for 72 hours at 37°C. The material was dialyzed overnight at 4°C against PBS, pelleted as above, resuspended in PBS or water to 5 mg protein ml⁻¹ and stored at -80°C. Protein was measured by a Coomassie Blue binding assay (Pierce, Rockford, IL, USA) for sodium hydroxide disrupted virus.

Preparation of the Vaccine with Mucoadhesives

Gels were prepared for all the mucoadhesives following simple methods available from the manufacturers. The sodium carboxymethyl cellulose (CMC) used for this experiment was the 7MF form and was purchased from Aqualon (Wilmington, DE). A CMC gel was prepared by making a 2% solution in H₂O. Carbopol and Polycarbophil acrylic polymers were purchased from B.F. Goodrich (Cleveland, Ohio). Gels were produced from these polymers by preparing a 0.25% suspension of Carbopol in H₂O, and a 0.5% suspension in Polycarbophil in H₂O. The pH of these solutions was raised from approximately 3 to approximately 4 by adding several drops of 1N NaOH. The acrylic polymer suspension becomes a gel when the pH reaches 4. Sodium alginate was purchased from Kelco, a division of Merck & Co., Inc. (San Diego, CA). A gel was produced by preparing a 2% solution in H₂O. Zilactin was purchased from

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Zila Pharmaceuticals, Inc. (Phoenix, AZ). A 1:10 solution of Zilactin to H₂O was prepared immediately before immunization.

Influenza vaccine solution (50 µg of virus protein/ 10 µl of phosphate buffered saline) was mixed with the mucoadhesive gels at a 1:50 ratio of vaccine to gel (10 µl vaccine solution and 490 µl mucoadhesive per dose). In all cases, mixing was accomplished by simple agitation for approximately 1-2 minutes, until a homogeneous solution was achieved by visual inspection, and was done immediately before immunization.

Immunization of Mice

Groups of five BALB/c mice (8 weeks old, female) obtained from Charles River or Jackson Laboratories were used. The vaccine in mucoadhesive (500 µl) was administered intragastrically with an animal feeding needle. For one control group the influenza vaccine, in 0.1 M NaHCO₃ solution, was also delivered intragastrically with an animal feeding tube.

Systemic immunization of one control group was performed with free antigen (50 µg/mouse) in a saline solution by subcutaneous route.

Collection of Samples

Blood was collected from the tail veins of mice before and at selected times after immunization. Blood was centrifuged and plasma was collected and frozen. Stimulated saliva was collected with capillary tubes after intraperitoneal injection of mice with carbamylcholine chloride (1 µg/mouse). Amounts of 2 µg each of soybean trypsin inhibitor, phenylmethyl-sulphonyl fluoride, sodium azide and fetal calf serum were added before clarification and storage at -80°C.

ELISA

For determination of antigen-specific antibodies, ELISA was performed in 96-well polystyrene microtiter plates (Dynatech, Alexandria, VA, USA) coated with purified A/Udorn influenza virus at a concentration of 4 µg ml⁻¹. Endpoint

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titers of serum and saliva were determined using horseradish peroxidase-labelled goat IgG against mouse Ig or IgA (Southern Biotechnology Associates, Birmingham, AL, USA) and substrate 2,2-azino-bis-(3-ethylbenzthiazoline) sulphonic acid (Sigma, St. Louis, MO, USA). The color developed was measured in a Vmax photometer (Molecular Devices, Palo Alto, CA, USA) at 414 nm.

Hemagglutinin Inhibition (HI) Assays

Hemagglutination inhibition (HI) assays were performed with mouse sera diluted 1:5 with PBS and treated for the removal of non-specific inhibitors (heated at 56°C for 30 minutes; incubated with 15% acid-treated kaolin for 30 minutes; and incubated with 10% suspension of chicken red blood cells for 30 minutes). Twofold dilutions of sera were prepared in 96-well microtiter plates. Viral suspension (8 HA units in an equal volume) was added to each well and incubated at room temperature for 30 minutes. A 0.5% suspension of chicken erythrocytes was added to each well and incubated at room temperature for 45-60 minutes. The HI titers were expressed as the reciprocal of the highest dilution that completely inhibited hemagglutination of erythrocytes.

Result

As shown in Table 1, all groups of mice that were immunized orally with a preparation of virus in mucoadhesive responded with higher serum immunoglobulin and hemagglutinin titers and higher titers of IgA in the saliva. The mucoadhesive carboxymethyl cellulose resulted in the best immune response in this example.

Table 1

	ELISA TITER				HI TITER IN SERUM	
	SERUM Ig		SALIVARY IgA			
	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
Oral control (with bicarbonate, no mucoadhesive)	8,000	32,000	10	40	10	<10
S.C. control (in PBS, no mucoadhesive)	8,000	512,000	10	10	<10	160
Carboxymethyl cellulose 2.0% (w/v) in H2O	16,000	512,000/ 1,024,000	<10	>80	<10	160
Carbopol 0.25% (w/v) in H2O, pH 4.0	8,000	128,000	<10	>80	<10	160
Polycarbophil 0.5% (w/v) in H2O, pH 4.0	8,000	256,000	<10	>80	<10	40
Sodium alginate 2.0% (w/v) in H2O	8,000	128,000	<10	>80	<10	40
Zilactin 1:10 (v/v) solution in H2O	8,000	64,000	<10	20	<10	<10
5 mice/group, assays performed on pooled sera Data report antibody titers in saliva (IgG by ELISA) and serum (total Ig by ELISA and hemagglutinin inhibition titer) both before and 28 days after immunization with 50µg of influenza A/Udorn (control only) either free or mixed with various mucoadhesives and administered by subcutaneous injection or by oral gavage.						

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EXAMPLE 3

To determine the concentration of mucoadhesive required for an optimal immune response, mice were immunized by oral gavage with the virus was prepared in various concentrations of the mucoadhesive carboxymethyl cellulose (substitution type 7MF). In addition, two groups of mice were immunized by oral gavage with influenza A/Udorn virus prepared in substituted carboxymethyl celluloses, either type 9M31FPH or 12M31P. Ten groups of mice, comprised of five, female BALB/c mice per group were immunized with the mucoadhesive preparation containing formalinized influenza virus (A/Udorn) prepared as described in Example 2, except that the preservative, thimerosal, was added to the allantoic fluid at a concentration of 0.02% (w/v) on harvesting of the virus and was maintained at the same concentration in all the subsequent steps of viral preparation. The mice were immunized, the serum collected and the hemagglutinin inhibition titers determined. Although positive serum hemagglutinin inhibition titers were obtained at day 14 and day 28 after immunization with the free virus subcutaneously, no serum hemagglutinin inhibition titers were obtained after oral immunization in the presence of concentrations of from 0.05% to 4.0% (w/v) of the mucoadhesive, carboxymethylcellulose. Salivary IgA and antibody levels by ELISA were not determined.

EXAMPLE 4

The effect of the contents of the stomach at the time of oral gavage on the outcome of the immunization was investigated by modifying the feeding regimen of the mice. Four groups of mice, comprised of five, female BALB/c mice per group, were placed on regimens in which they were deprived of food or water prior to immunization by oral gavage with a preparation containing 2% (w/v) carboxymethyl cellulose mucoadhesive and 50 µg of a preparation of

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formalinized influenza virus A/Udorn, prepared as described in Example 3. That is, the virus had been prepared in the presence of preservative (0.02% thimerosal). The mice were immunized, bled and the hemagglutination titer of the serum determined as described in Example 2. No serum hemagglutinin inhibition titers were obtained at day 14 in any of these groups, including the control group that were offered food and water *ad libitum*. Salivary IgA and antibody levels by ELISA were not determined.

EXAMPLE 5

Example 2 indicates that oral immunization with influenza virus (A/Udorn) prepared according to the protocol described in Example 2 yielded positive serum hemagglutinin titers on immunization by oral gavage in the presence of mucoadhesives, including carboxymethyl cellulose. In contrast, Examples 3 and 4 indicate that influenza A/Udorn virus prepared according to Example 3 failed to yield positive serum hemagglutinin titers on oral immunization in the presence of carboxymethyl cellulose, but did yield positive serum hemagglutinin titers on subcutaneous immunization with the free virus. The protocol used in the preparation of the influenza virus according to Example 3, included the use of the preservative, thimerosal. The omission of this preservative during the preparation of the virus as described in Example 2 could have resulted in bacterial contamination of the viral stocks. Therefore, the viral stocks were cultured to determine if bacterial contamination was present. Stocks prepared in the presence of thimerosal were found to be free of bacterial contamination, whereas a viral stock prepared in the absence of thimerosal was found to be contaminated with bacteria. Detailed analysis of this viral preparation identified the bacterial contamination as *Klebsiella planticola* and *Xanthomonas maltophilia*.

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The influenza virus A/Udorn/307/72 (H3N2), BK6, Egg3, clone 3A (7-25-89) containing *Klebsiella planticola* and *Xanthomonas maltophilia* was deposited with the American Type Culture Collection, Rockville, Maryland.

EXAMPLE 6

The effect of bacterial contamination of the viral stock on the outcome of the immunization in the presence of carboxymethyl cellulose was determined by comparing immunization of the mice with stock prepared according to Example 3 and known to be free of contamination and the stock known to be contaminated with the bacteria, *Klebsiella planticola* and *Xanthomonas maltophilia*. Mice were immunized, bled and the serum hemagglutinin inhibition titer and serum anti-influenza total immunoglobulin antibodies assayed by ELISA determined, all as described in Example 2.

Results

As shown in Table 2, the influenza virus stock that was prepared according to Example 2 in the absence of thimerosal and known to be contaminated with bacteria was effective in eliciting an immune response in the mice as assayed by ELISA or serum hemagglutinin inhibition when delivered either subcutaneously or orally. The immune response elicited by oral immunization in the presence of the mucoadhesive and the bacterial contaminant was higher than that elicited by the immunogen containing the bacterial contaminant in the absence of the mucoadhesive, indicating that both the bacterial contaminant and the mucoadhesive were required for the effective elicitation of an immune response. In contrast, although the virus stock prepared with thimerosal according to Example 3 that was known to be free of bacterial contamination was capable of eliciting an immune response on subcutaneous immunization, it was unable to elicit a significant immune response on oral immunization even in the presence of the mucoadhesive, carboxymethyl cellulose. Moreover, when the results obtained in the ELISA assay for Groups 1, 2 and 4 are combined and the sum compared with the

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ELISA results for Group 5, it appears that synergism occurred between the immunogen, adjuvant, and mucoadhesive according to the invention.

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Table 2

Group #	IMMUNOGEN	ELISA ASSAY		SERUM HEMAGGLUTININ INHIBITION TITER	
		Day 0	Day 28	Day 0	Day 28
Bacteria-free virus stock prepared according to Example 2 in the presence of thimerosal					
1	Oral immunization with 50 μ g influenza virus with 500 μ l NaHCO ₃	16,000	16,000	<4	<4
2	Oral immunization with 50 μ g influenza virus with carboxymethylcellulose	16,000	32,000	<4	<4
3	Subcutaneous immunization with 50 μ g influenza virus	16,000	128,000	<4	32/64
Bacterial-contaminated stock prepared according to Example 1 in the absence of thimerosal.					
4	Oral immunization with 50 μ g influenza virus with 500 μ l NaHCO ₃	8,000	32,000	<4	<4
5	Oral immunization with 50 μ g influenza virus with carboxymethylcellulose	8,000	128,000	<4	64

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Table 2 (Cont'd)

Group #	IMMUNOGEN	ELISA ASSAY		SERUM HEMAGGLUTININ INHIBITION TITER	
		Day 0	Day 28	Day 0	Day 28
6	Subcutaneous immunization with 50 μ g influenza virus	16,000	128,000	<4	128
<p>5 mice/group, assays performed on pooled sera</p> <p>Data report antibody titers in serum (total Ig by ELISA and hemagglutinin inhibition titer) both before and 28 days after immunization with 50 μg of influenza A/Udorn (control only) either prepared in the absence of thimerosal and known to be contaminated with bacteria (protocol of Example 1) or prepared in the presence of thimerosal and known to be free of bacterial contamination (protocol of Example 2). The virus was free or mixed with the mucoadhesive carboxymethyl cellulose, substitution type 7MF (2% w/v in H₂O) and administered by subcutaneous injection or by oral gavage.</p>					

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EXAMPLE 7

As indicated in Example 6, viral preparations that were known to be contaminated with bacteria were capable of eliciting an immune response when delivered orally in the presence of the mucoadhesive, carboxymethyl cellulose. In contrast, viral preparations that were not contaminated with bacteria did not elicit an immune response when delivered orally in the presence of the mucoadhesive, carboxymethyl cellulose. The possibility that the bacteria functioned as an adjuvant in Example 6 was tested by including other known adjuvants in the formulation. The effect of the adjuvants lipopolysaccharide and muramyl dipeptide in the presence of the mucoadhesive, carboxymethylcellulose, was therefore determined. Mice were immunized orally using 50 μ g of an influenza virus preparation that was known to be free of bacteria with 500 μ l of 2% carboxymethyl cellulose, either with or without the addition of LPS or MDP as an adjuvant.

Results

As shown in Table 3, the addition of LPS derived from either *Klebsiella pneumoniae* or *Escherichia coli* to the carboxymethyl cellulose mucoadhesive preparation resulted in the elicitation of a positive immune response as assayed by serum hemagglutinin titers or by assay of salivary IgA titers. Addition of muramyl dipeptide also resulted in the elicitation of an immune response as assayed by serum hemagglutinin titer or salivary IgA, although the levels of salivary IgA titers were slightly lower than those obtained with LPS.

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Table 3

Group #	IMMUNOGEN	SERUM HEMAGGLUTININ INHIBITION TITER			SALIVARY IgA TITER	
		Day 0	Day 14	Day 28	Day 0	Day 28
Subcutaneous immunization						
1	50 µg influenza virus	<4	128	256	<10	10
Oral immunization with carboxymethylcellulose and lipopolysaccharide or muramyl dipeptides						
2	50 µg influenza virus with 500 µl of 2% (w/v) CMC	<4	<4	<4	<10	<10
3	50 µg influenza virus with 500 µl of 2% (w/v) CMC with 1 mg LPS (<i>Klebsiella pneumoniae</i>)	<4	64	64	<10	>80
4	50 µg influenza virus with 500 µl of 2% (w/v) CMC with 1 mg LPS (<i>Escherichia coli</i>)	<4	128	128	<10	>80

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CLAIMS

What is claimed is:

1. An immunization composition comprising an immunizing amount of an antigen and a mucoadhesive in an amount sufficient to induce or enhance immune response to the antigen.

2. Composition as claimed in claim 1, wherein the antigen is an influenza virus antigen.

3. Composition as claimed in claim 1, wherein the mucoadhesive is selected from the group consisting of sodium carboxymethyl cellulose and other cellulose derivatives, polycarbophil, and carbopol.

4. An immunization composition comprising (a) an immunizing amount of an antigen, and (b) a mucoadhesive and an adjuvant in an amount sufficient to induce or enhance immune response to the antigen.

5. Composition as claimed in claim 4, wherein the antigen is an influenza virus antigen.

6. Composition as claimed in claim 4, wherein the mucoadhesive is selected from the group consisting of sodium carboxymethyl cellulose and other cellulose derivatives, polycarbophil, and carbopol.

7. Composition as claimed in claim 4, wherein the adjuvant is selected from the group consisting of muramyl peptide, squalene, saponin, and the monophosphoryl derivative of lipid A.

8. A method of immunizing an animal, wherein the method comprises administering to the animal an immunizing amount of the composition of claim 1.

9. The method as claimed in claim 8, wherein the antigen is an influenza virus antigen.

10. The method as claimed in claim 8, wherein the mucoadhesive is selected from the group consisting of sodium

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carboxymethyl cellulose and other cellulosics, polycarbophil, and carbopol.

11. The method as claimed in claim 8, wherein the immunization composition is administered orally, nasally, rectally, or swab on the tonsil.

12. A method of immunizing an animal, wherein the method comprises administering to the animal an immunizing amount of the composition of claim 4.

13. The method as claimed in claim 12, wherein the antigen is an influenza virus antigen.

14. The method as claimed in claim 12, wherein the mucoadhesive is selected from the group consisting of sodium carboxymethyl cellulose and other cellulosics, polycarbophil, and carbopol.

15. The method as claimed in claim 12, wherein the adjuvant is selected from the group consisting of muramyl peptide, squalene, saponin, and the monophosphoryl derivative of lipid A.

16. The method as claimed in claim 12, wherein the immunization composition is administered orally, nasally, rectally, or swab on the tonsil.

17. A method of immunizing an animal, wherein the method comprises orally administering to the animal an immunizing amount of the composition of claim 4.

18. The method as claimed in claim 17, wherein the antigen is an influenza virus antigen.

19. The method as claimed in claim 18, wherein the antigen is administered to the animal in an enteric coated dosage unit.

20. The method as claimed in claim 19, wherein the antigen, mucoadhesive, and adjuvant are simultaneously administered to the animal.

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 94/02454

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 A61K9/00 A61K47/32 A61K47/38

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 230 264 (BEHRINGWERKE AKTIENGESELLSCHAFT) 29 July 1987 see the whole document see column 2, line 42 - line 49 see column 3; examples 1,2 ---	1,3,8, 10,11
A	DEUTSCHE APOTHEKER ZEITUNG vol. 131, no. 8, 21 February 1991, STUTTGART (DE) pages 295 - 303 XP173519 H.E. JUNGINGER ET AL. 'nasale arzneiformen für die systemische wirkstoffabsorption' see page 302, column 3 --- -/--	1-20



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

28 June 1994

Date of mailing of the international search report

04. 07 94

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 94/02454

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 391 342 (TOKO YAKUHI KOGYO KABUSHIKI) 10 October 1990 see page 2, line 1 - page 9, line 44 see page 6, line 1 see page 13, line 18 - page 17, line 24 ---	1-20
X	EP,A,0 122 036 (TEIJIN LIMITED) 17 October 1984 see page 22; example 15 ---	1-3,8-11
X	EP,A,0 304 786 (MOBAY CORPORATION) 1 March 1989 see page 4, line 22 - line 45 see page 5 - page 6; example 1 see claims 1,7,8 ---	1-20
A	EP,A,0 283 085 (AKZO N.V.) 21 September 1988 ---	
P,X	WO,A,94 02170 (PURDUE RESEARCH FOUNDATION) 3 February 1994 see the whole document -----	1,19

INTERNATIONAL SEARCH REPORT

Inter. application No.

PCT/US 94/ 02454

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 8-20 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the composition (Rule 39.1iv PCT).
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 94/02454

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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